Examining Interactions of HIV-1 Reverse Transcriptase with Single-stranded Template Nucleotides by Nucleoside Analog Interference*

Received for publication, April 25, 2006, and in revised form, June 28, 2006 Published, JBC Papers in Press, July 25, 2006, DOI 10.1074/jbc.M603970200

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Crystallographic studies have implicated several residues of the p66 fingers subdomain of human immunodeficiency virus type-1 reverse transcriptase in contacting the single-stranded template overhang immediately ahead of the DNA polymerase catalytic center. This interaction presumably assists in inducing the appropriate geometry on the template base for efficient and accurate incorporation of the incoming dNTP. To investigate this, we introduced nucleoside analogs either individually or in tandem into the DNA template ahead of the catalytic center and investigated whether they induce pausing of the replication machinery before serving as the template base. Analogs included abasic tetrahydrofuran linkages, neutralizing methylphosphonate linkages, and conformationally locked nucleosides. In addition, several Phe-61 mutants were included in our analysis, based on previous data indicating that altering this residue affects both strand displacement synthesis and the fidelity of DNA synthesis. We demonstrate here that altering the topology of the template strand two nucleotides ahead of the catalytic center can interrupt DNA synthesis. Mutating Phe-61 to either Ala or Leu accentuates this defect, whereas replacement with an aromatic residue (Trp) allows the mutant enzyme to bypass the template analogs with relative ease.

The structures of unliganded, inhibitor-bound, and nucleic acid-containing human immunodeficiency virus type 1 reverse transcriptase (HIV-1 RT)4 have been instrumental in dissecting the multifunctional properties of this key retroviral enzyme and exploiting it as a target for antiviral therapy (1–6). In particular, co-crystals of RT with duplex DNA (2, 7) and an RNA/DNA hybrid (6) have highlighted critical contacts mediated by protein motifs of its N-terminal DNA polymerase and C-terminal ribonuclease H domains. Examples of such motifs include the “translocation track” (8, 9) and primer grip of the p66 thumb subdomain (2), the template grip (10), and a second primer grip in the vicinity of the ribonuclease H catalytic center (6). Roles for these motifs have been investigated in vitro by site-directed mutagenesis (8, 9, 11–20) and complemented by studying the effects of strategic placement of nucleoside analogs into either the template or primer (21–28). In parallel, replication of viruses harboring mutations in these motifs have been extensively examined in vivo (17, 29–31).

Although a number of studies have investigated the geometry of duplex nucleic acid positioned between the catalytic centers of HIV-1 RT, the manner in which the single-stranded template traverses the p66 fingers subdomain before accessing the DNA polymerase catalytic center is less well understood. Stated differently, when is the relatively disordered, single-stranded nucleic acid oriented to serve as the template base for accurate and efficient incorporation of the incoming dNTP? Although less precise in the resolution they provide, enzymatic footprinting and photocross-linking studies (32, 33) suggest template nucleotides as far as position +7 (defining n + 1 at the first unpaired template nucleobase and n as the adjacent, nascent base pair) could contact the p66 fingers of the enzyme. The model of Huang et al. (7) indicates that template nucleobases +2 and +3 pack against Trp-24, Pro-25, Phe-61, and Ile-63 of the p66 fingers. In addition, Leu-74 contacts template nucleobase n + 1, whereas phosphate contacts involve Arg-78 (n + 1/n), Lys-154 (n/n − 1) and Glu-89 (n − 1/n − 2) (Fig. 1A). Based on this model, site-directed mutagenesis has demonstrated that substituting Phe-61 alters fidelity (34), processivity, and strand displacement synthesis (35). The focus of the present study was to determine whether (a) altering the nucleobase, sugar, or phosphate backbone of the 5’ template overhang ahead of the polymerase catalytic center influences orientation of the template base and (b) how this is affected by substitution of amino acids implicated in such interactions.

The strategy we pursued is based on our recent analysis of (+) strand DNA synthesis from the polypurine tract primer of retroviruses (25, 26, 28) and long terminal repeat-containing retrotransposons (36, 37). In these studies nucleoside analogs altering duplex geometry were exploited to define regions of the polypurine tract-containing RNA/DNA hybrid interacting with the cognate RT. In the present investigation nucleoside analogs were inserted either individually or in tandem into the

‡This work was supported in part by the Intramural Research Program of the National Institutes of Health, NCI, and Center for Cancer Research (to C. D. and S. Le G.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
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4The abbreviations used are: HIV-1, human immunodeficiency virus, type 1; LNA, locked nucleic acid; nt, nucleotide(s); RT, reverse transcriptase; THF, tetrahydrofuran.

Printed in the U.S.A.
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5′ template overhang ahead of the DNA polymerase catalytic center. By doing so, our goal was to investigate whether such perturbations induced stalling of the replication complex and determine the distance from the template base at which this event occurred. Locked nucleic acid (LNA) analogs (38) were selected to impart rigidity into the template backbone through the conformationally constrained sugar ring. In contrast, methylphosphonate linkages were introduced to locally neutralize the phosphate backbone and probe the involvement of electrostatic interactions (39, 40). Last, abasic tetrahydrofuran (THF) linkages (41–43) were introduced as a means of locally eliminating the nucleobase while preserving the sugar-phosphate backbone of the template. Re-positioning the DNA primer relative to the site of analog insertion allowed DNA synthesis to be evaluated under running start and standing start conditions. Collectively, our data indicate that altering template geometry 1–2 nucleotide (nt) ahead of the catalytic center influences protein-nucleic acid contacts to stall HIV-1 RT and that this effect can be modulated by Phe-61 substitutions.

EXPERIMENTAL PROCEDURES

Design, Synthesis, and Purification of Oligodeoxynucleotides—A 45-nt DNA template (5′-TAC ATA CCC ATA CAT AAA TCC TAA CCT TGA AGA ACT CGT CAC 5′) was designed with minimal secondary structure, as determined by the mFOLD program (44); bases underlined and bold indicate the positions of single, double, or triple substitutions of nucleoside analogs. Phosphoramidites of d-Spacer and methylphosphonates were purchased from Glen Research, Sterling, VA, and LNA phosphoramidites were from Proligo Reagents, Boulder, CO. Analog-substituted oligonucleotide templates were synthesized at a 1 μmol scale on a PE Biosystems Expedite 8909 nucleic acid synthesizer by standard phosphoramidite chemistry. Stepwise coupling yields for incorporation of the analogs were >98% as determined by trityl cation monitoring. Sites of substitution are illustrated in Figs. 1, C and D, and are described further under “Results.” Deprotection and cleavage of the DNA templates from the Controlled Pore Glass support were carried out by incubation in 30% NH₄OH for 36 h at 25 °C. Methylphosphonate-substituted oligonucleotides were deprotected according to the manufacturer’s modified protocol. Oligonucleotides were purified by preparative polyacrylamide gel electrophoresis and quantified spectrophotometrically (260 nm), assuming a molar extinction coefficient equal to the sum of the constituent deoxynucleotides. DNA primers were designed to mimic initiation and elongation of DNA synthesis. Primers with the following sequences are also synthesized and purified as per the protocol described above: primer for running start DNA synthesis, 5′-TAT GGG TAT GTA TTT AGG ATT-3′; primer for standing start DNA synthesis, 5′-ATG TAT TTA GGA TTG GAA CTT-3′.

Expression and Purification of Recombinant Proteins—Wild type HIV-1HXB2 RT and enzymes containing Phe-61 substitutions were transformed into the E. coli strain DH5αF’IQ (Invitrogen) for protein expression. To purify heterodimeric RTs with mutations only within the p66 subunit of RT, the p66 and p51 subunits were expressed separately. Plasmid p6HRT51 was used to express wild type p51, which contains an N-terminal Hisᵥ tag to facilitate purification of heterodimers via Ni²⁺-nitritoltriacetic acid chromatography (45). After induction of p66 and p51 subunits, bacterial cell pellets were combined before cell lysis and further purification (46). Enzymes were initially purified by a combination of metal chelate (Ni²⁺-NTA-Sepharose, Qiagen Inc., CA) and size exclusion chromatography (Superdex-200, GE Healthcare). The latter purification step was conducted in a buffer containing 50 mM sodium phosphate buffer (pH 7.8), 0.7 M NaCl. Purified enzymes were stored at −20 °C in 50% glycerol containing 50 mM sodium phosphate buffer (pH 7.8), 0.7 M NaCl. Under these conditions we observed minimal loss of activity over several months.

DNA-dependent DNA Polymerase Activity—DNA synthesis was measured on 45-nt DNA templates annealed to a 5′ end-labeled DNA primer. Polymerization was initiated by adding 10 nM enzyme to a mixture containing 50 nM template/primmer, 200 μM dNTPs, 25 mM Tris-HCl (pH 7.8), 80 mM NaCl, 6 mM dithiothreitol, and 9 M MgCl₂ at 37 °C and terminated after 5 min by adding an equal volume of a formamide-based gel-loading buffer. Polymerization products were fractionated by high voltage electrophoresis through denaturing 15% (w/v) polyacrylamide gels containing 7 M urea in 89 mM Tris borate (pH 8.3), 2 mM EDTA. After drying, gels were subjected to autoradiography or phosphorimaging analysis using Molecular Imager FX phosphorimaging (Bio-Rad) and quantified using Quantity One software (Bio-Rad).

Steady-state Kinetics of Polymerization—Steady-state kinetic parameters Vₘₐₓ and Kₘ were determined using the wild type and mutant template primer with the corresponding dNTP as the substrate as described by Boosalis et al. (47). DNA synthesis was initiated at 37 °C using variable concentrations of the corresponding dNTP. Reactions were stopped by mixing with an equal volume of Tris borate-EDTA and 95% (v/v) formamide containing 0.1% (w/v) bromphenol blue and xylene cyanol. Polymerization products were resolved by high voltage denaturing 10% PAGE and analyzed using phosphorimaging. Velocities for each substrate concentration were fit to a Michaelis-Menten plot to determine the Kₘ and Vₘₐₓ values. kₗₜₐₜ values were determined from the equation Vₘₐₓ = kₗₜₐₜ [E]ₜₒₜₐₜ.

RESULTS

Experimental Rationale—Our experimental strategy (Fig. 1) involved placing nucleoside analogs individually or in tandem into the single-stranded template overhang ahead of the DNA polymerase catalytic center. If an alteration in template geometry was required to correctly orient the template base as it traversed the p66 finger subdomain toward the active site, we reasoned that locally disrupting this geometry would interrupt translocation before that analog accessing the active site. Nucleoside analogs imparting different properties were exploited for this strategy (Fig. 1B). LNA analogs, which locally increase organization of the sugar-phosphate backbone, were used to enhance stacking and decrease flexibility (38). In contrast, THF deoxyriboside linkages lack the nucleobase, thereby decreasing stacking and increasing flexibility of the phosphate backbone (41). At the same time, these analogs serve as probes of protein/nucleobases interactions. Finally methylphosphonate linkages locally neutralize the negative charge of the phospho-
phate backbone and were used to identify the contribution of electrostatic interactions (39, 40, 48). Polymerization was evaluated on two DNA duplexes. In the substrate of Fig. 1, the analogs occupy positions \( n/8 \) (G), \( n/8/9 \) (GA), or \( n/8/9/10 \) (GAA) and assess DNA synthesis under “running start” conditions (49). Alternatively, an equivalently sized primer was hybridized immediately adjacent to the site of analog insertion (Fig. 1D) to assess the same structural anomalies under standing start conditions (50). Finally, based on a proposed interaction of p66 fingers residue Phe-61 with the template overhang (7), the DNA synthesis properties of several selectively mutared Phe-61 variants (34, 35) were investigated.

Polymerization Properties of Wild Type HIV-1 RT on Modified Templates—DNA polymerase activity of wild type HIV-1 RT under running start conditions is illustrated in Fig. 2, A–C. An 8-nt primer extension product (i.e. \( p + 8 \)) on a substrate containing an \( n + 8 \) template THF lesion indicated efficient incorporation opposite the lesion (Fig. 2A, lane 1), although in contrast to Cai et al. (51), subsequent mispair extension was inefficient. In addition, \( p + 6 \) and \( p + 7 \) pause sites were
observed. According to Huang et al. (7), p66 residue Leu-74 contacts nucleobase \( n + 1 \), whereas Phe-61 contacts the \( n + 1 \) ribose and \( n + 2 \) nucleobase (Fig. 1A). Pausing two nucleotides before the lesion accessing the catalytic center (\( p + 6 \) product) most likely reflects loss of contact between Phe-61 and the \( n + 2 \) template nucleobase as a result of the THF lesion. Further extending the primer by one nucleotide relocates the lesion to occupy what would otherwise be template nucleobase \( n + 1 \). In this case, although \( n + 2 \) contact with Phe-61 is restored, the lesion now eliminates contact with Leu-74, resulting in the \( p + 7 \) primer extension product.

Surprisingly, an \( n + 8/n + 9 \) THF lesion significantly reduced \( p + 8 \) synthesis and concomitantly increased that of the \( p + 7 \) product (Fig. 2A). Quantification (data not shown) indicated that a 3–4-fold reduction in the former was accompanied by a \( \sim 2 \)-fold increase in the latter, and a similar trend was induced by the triple THF substitution (Figs. 2A, panel 3). The structural model summarized in Fig. 1A predicts that when DNA synthesis positions two consecutive template THF lesions in front of the active site, contact with nucleobases \( n + 1 \) (Leu-74) and \( n + 2 \) (Phe-61) will be simultaneously eliminated, which our data suggest either prevents correct positioning of the incoming dNTP opposite the first of these or induces the dNTP to bind in an orientation unfavorable for catalysis. The similarity in DNA synthesis profiles between dual- and triple-substituted templates suggests a contribution from nucleobase \( n + 3 \) is not mandatory.

LNA analogs (Fig. 2B) did not completely interrupt translocation but induced a major pause site at \( p + 6 \). Such a result suggests LNA-induced structural changes in the template overhang were also “sensed” by residues of the fingers subdomain 2 nt ahead of the catalytic center. The same phenotype was observed when the experiment of Fig. 2B was conducted in the presence of the competitor heparin (data not shown), implying the replication complex does not dissociate after pausing and that enzyme stalled at this position can gradually bypass the structural anomaly. The model of Huang et al. (7) suggests that p66 residue Trp-24 interacts with the phosphate backbone between nucleobases \( n + 2 \) and \( n + 3 \) of the template backbone. Because LNA analogs re-organize the phosphate backbone due to their C3′-endo sugar pucker, this appears to invoke a steric clash that transiently stalls the translocation complex on the template 2 nt before the catalytic center. However, the equivalent response independent of the extent of LNA substitution was unexpected. One possible explanation might be that, because RT pauses without dissociation, the ternary complex re-establishes the conformation of a standing start complex, which is insensitive to LNA substitution. Data of a later section indeed support this notion. To eliminate the possibility that LNA-induced pausing was a feature of the template sequence used in this work, a DNA polymerase experiment was performed on an unrelated duplex containing \( +5/\pm 4 \) (3′-CA-5′) and \( +4/\pm 3 \) (3′-AG-5′) LNA insertions. Under these conditions pausing was observed at positions \( p + 2 \) and \( p + 1 \), respectively, i.e. again, two nucleotides before the LNA was encountered by the DNA polymerase active site (data not shown).

Fig. 2C examines the effect of neutralizing the phosphate backbone of the template overhang. Regardless of the extent of substitution, the primary pause site was after incorporation opposite template nucleobase \( n + 8 \), with limited synthesis thereafter. In this paused complex, Lys-154 will contact the template backbone between the nascent \( n \) and penultimate base pair \( (n - 1) \) of the template-primer duplex (Fig. 1A and Ref. 7). dNTP incorporation opposite template nucleobase \( n + 8 \) has the consequence that the template \( n + n = \) 1 phosphodiester linkage is neutralized, which we believe compromises the interaction with Lys-154 to transiently disrupting DNA synthesis, although we cannot rule out that local alteration of duplex geometry (39) may also re-orient the primer terminus relative to the incoming dNTP. A dual template methylphosphonate linkage further reduces polymerization after incorporation opposite template nucleobase \( n + 8 \). Because Arg-78 contacts the template \( n/n + 1 \) phosphate (7), we interpret this phenotype as simultaneous loss of contact to Lys-154 and Arg-78 further aggravates polymerization. Introducing three consecutive methylphosphonate linkages led to almost complete arrest of DNA synthesis beyond nucleobase \( n + 8 \), suggesting additional contacts between the single-stranded template backbone and residues of the p66 fingers subdomain were affected.

Properties of Phe-61 HIV-1 RT Mutants on Modified Templates—p66 fingers residue Phe-61 of HIV-1 RT has been implicated in contacting the template overhang immediately ahead of the catalytic center, and mutation of this residue is associated with alterations in fidelity, nucleoside analog sensitivity, and strand displacement synthesis (7, 34, 35). We, therefore, elected to investigate how F61A, F61L, and F61T mutants were affected as template analogs accessed their DNA polymerase active site.

Regardless of the number of template THF lesions encountered, F61A and F61L RT terminated DNA synthesis after incorporation opposite nucleobase \( n + 7 \) (Fig. 3A, panels i and ii, respectively). If, as proposed, Phe-61 contacts template \( n + 1 \) ribose and nucleobase \( n + 2 \) (Fig. 1A), we assume both would be compromised by introducing Ala, predicting DNA synthesis would be interrupted when the abasic lesion occupied position \( n + 1 \) and not the catalytic center, i.e. after \( p + 7 \) synthesis. This model would also predict that, once the primary contact with Phe-61 was lost, introducing dual or triple template lesions would likewise stall the mutant enzyme at position \( p + 7 \), which we observed experimentally. Although replacing Phe-61 with Leu introduces a residue with a longer side chain, data of Fig. 3A, panel ii, suggests this does not restore contact with template nucleobases, since again translocation is interrupted after incorporation opposite template nucleobase \( n + 7 \). Consistent with the need for an aromatic residue at this position for optimal packing against template nucleobases (7), mutant F61T will incorporate a dNTP opposite the first THF lesion at position \( n + 8 \) and to a lesser extent at \( n + 9 \) (Fig. 3A, panel iii). As noted for wild type RT, consecutive THF lesions also induce these mutants to stall after dNTP incorporation opposite nucleobase \( n + 7 \).

Fig. 3B depicts DNA synthesis by mutant enzymes over LNA-substituted templates. As noted for the parental enzyme, F61A...
and F61L RT stalled after incorporation opposite nucleobase n + 6, although the analog was present at position n + 8, strengthening our notion that constraining the template backbone 2 nt ahead of the catalytic center alters contact with the fingers subdomain (Fig. 3B, panels i and ii). However, in this case consecutive LNA insertions progressively impaired DNA synthesis such that stalling at p + 6 predominated on a triple-substituted template. In contrast, F61T RT was virtually insensitive to the LNA-induced translocation barrier regardless of the extent of substitution (Fig. 3B, panel iii). Although the requirement for an aromatic residue at position 61 of the p66 fingers subdomain is clear from these experiments, bypassing the LNA substitutions was unexpected. Modeling studies (35)

proposed that introducing Trp for Phe-61 could provide increased contact to template nucleobase n + 2, which the data of Fig. 3B, panel iv, imply can compensate for a steric clash imposed by LNA-induced re-organization of the phosphate backbone.

F61A RT stalled primarily opposite nucleobase n + 7 on a substrate containing an n + 7/n + 8 methylphosphonate substitution (Fig. 3C, panel i). This likely reflects two events, namely (a) loss of contact normally established between the Phe-61 side chain and the ribose of the first unpaired template nucleobase and (b) methylphosphonate-induced loss of contact between Arg-78 and the template n/n + 1 phosphate. The phenotype of F61L RT is essentially the same as wild type RT (Fig. 3C, panel ii). However a single methylphosphonate linkage failed to inhibit F61T RT (Fig. 3C, panel iii), implying increased aromaticity can compensate for limited neutralization of the phosphate backbone. As the extent of neutralization increases, F61T RT pauses after incorporation opposite template nucleobase n + 8 (dual-substituted template) or n + 8 and n + 9 (triple-substituted template). Although the rationale for pausing opposite n + 8 is similar to that described for wild type RT, incorporation opposite two methylphosphonate linkages will affect contact with Glu-89 (n − 2/n − 1 phosphate), Lys-154 (n − 1/n phosphate), and Arg-78 (n + 1/n + 2 phosphate). However, we cannot rule out the alternative that the geometry of the nascent duplex containing two methylphosphonate linkages changes in response to asymmetric distribution of charge on its backbone.

**FIGURE 3.** DNA polymerase activity of Phe-61 HIV-1 RT mutants on template variants containing THF lesions (A), LNA analogs (B), and methylphosphonate linkages (C) between positions n + 8, n + 9, and n + 10. The p66 Phe-61 variant is indicated below each panel. The lane notations (0, 1, 2, and 3) above each panel represent the number of analog insertions. P, unextended.

**FIGURE 4.** DNA polymerase activity of wild type HIV-1 RT on template variants containing THF lesions (A), LNA analogs (B), and methylphosphonate linkages (C) at positions n + 1, n + 2, and n + 3. The numbers (1, 2, and 3) above each panel again represent the number of analog insertions; 0 indicates the unsubstituted template. The migration positions of the unextended (P), fully extended (p + 11), and partially extended primer (p + 1) are indicated.
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base $n + 1$ as well as Phe-61 and template nucleobase $n + 2$ contribute to the architecture of the dNTP binding pocket. Regardless of the extent of LNA substitution, primer extension was unaffected (Fig. 4B). Because LNA substitutions at and beyond template nucleobase $n + 8$ induced stalling opposite nucleobase $n + 6$ under running start conditions (Fig. 2B), this predicted that a triple-substituted template would induce pausing at position $p + 1$, i.e. where the analog corresponded to nucleobase $n + 2$. One possible explanation for the data of Fig. 4B is that HIV-1 RT adopts different conformations during standing and running start conditions, which would be in keeping with some of our original studies (52–55). The same argument would explain the data of Fig. 2B, i.e. LNA-induced pausing effectively reestablishes as a standing start complex, which can bypass the triple LNA substitution. Last, methylphosphonate template substitutions induced the same phenotype during standing start DNA synthesis (Fig. 4C) as noted with running start conditions (Fig. 2C), i.e. progressively neutralizing the phosphate backbone compromises interactions with Lys-154 and Arg-78 and stalls the replication machinery.

Standing Start Mode of DNA Synthesis by Mutant Enzymes—DNA synthesis under standing start conditions on substituted duplexes by Phe-61 variants is shown in Fig. 5. As predicted, regardless of the extent of THF substitution, F61A and F61L RT barely initiated DNA synthesis (Fig. 5A, panels i and ii). Because under running start conditions the same mutants failed to synthesize opposite a THF lesion, data obtained with both template/primer duplexes suggest reduced affinity for the incoming dNTP or an incorrect orientation once bound. F61T RT efficiently incorporated opposite the initial THF lesion, whereas dual and triple insertions lower the efficiency of $p + 1$ synthesis (Fig. 5A, panel iii). The distal effect of THF lesions is particularly pronounced with F61T RT, where densitometry (not shown) indicated an ∼5-fold reduction in $p + 1$ synthesis with a dual- or triple-substituted template. Gel-mobility shift experiments using the three template-primer duplexes of Fig. 1C indicated that the stability of the binary complex was unaffected (data not shown).

Although F61A and F61L RT were unaffected by an $n + 1$ LNA insertion, polymerization efficiency decreased as the extent of substitution increased and was more pronounced with F61A RT (Fig. 5B, panels i and ii). With these two mutants, accumulation of a $p + 1$ product on a triple-substituted template suggests the ternary complex stalled after adding the first dNTP to the primer terminus. This affect was alleviated with F61T RT (Fig. 5B, panel iii), which like wild type enzyme, extended the primer to the template 5’-terminus regardless of the extent of substitution. Finally, methylphosphonate substitutions of the template immediately ahead of the catalytic center induced a common feature in that significant pausing at position $p + 1$ was observed for all mutants on an $n + 1$-substituted template (Fig. 5C). Although the DNA synthesis profiles for F61A and F61L RT in Fig. 3C predicted these two mutants would pause after incorporating a dNTP opposite the methylphosphonate linkage, this was unexpected for mutant F61T, which bypassed a single lesion under running start conditions (Fig. 3C, panel iii).

Kinetic Analysis—Of the nucleoside analogs employed, the phenotype induced by THF lesions was particularly interesting, i.e. an $n + 2$ template lesion was capable of altering events at the catalytic site. To investigate this in more detail, a series of single nucleotide addition experiments was performed. The substrates of Fig. 6, A–C, evaluated incorporation of dTTP opposite a template $n + 1$ adenosine when this is followed by a single or dual THF lesion. For wild type and F61T RT the $K_m$ for dTTP was not significantly affected by THF substitution, varying from 1.07 to 2.35 mM (Fig. 6). Taking $V_{max}$ into account, this represented a 2.6-fold reduction in catalytic efficiency for wild type RT on the dual-substituted template. For mutant F61T, catalytic efficiency was reduced by the same order of magnitude, i.e. 4.6-fold. These differences appear more related to catalysis rather than dNTP binding, which if anything appeared to be enhanced by nucleobase removal. In contrast, mutants F61A and F61L exhibited a 12.4- and 24.2-fold reduction in catalytic efficiency on the dual-substituted template, with the difference
coming primarily from reduced affinity for the incoming dNTP. An electrophoretic gel mobility shift analysis with the substrates indicated in Figs. 6, A–C, indicated no difference in affinity between wild type HIV-1 RT and the three Phe-61 mutants (data not shown). Taken together, the data of Fig. 6 provide supporting evidence that correct template geometry 2 nt ahead of the DNA polymerase active site is important for efficient catalysis.

**DISCUSSION**

We have exploited nucleoside analogs to investigate whether altering the nucleobase, sugar, or phosphate backbone of the single-stranded template overhang affects how the template base accesses the active site of HIV-1 RT for accurate and efficient catalysis. Several scenarios might be envisaged using this approach. Termination of DNA synthesis after incorporation opposite the analog implies that contacts in the vicinity of the nascent base pair and active site residues were compromised. Alternatively, synthesis terminated immediately before encountering the analog might suggest the template base is improperly positioned at the catalytic center, preventing correct docking of the incoming dNTP. A third scenario might be that polymerization terminates several bases before the modified template enters the active site, indicating an unfavorable interaction with residues of the p66 fingers subdomain required for maintaining active site geometry. Our data provides evidence for each of these scenarios during HIV-1 RT-catalyzed DNA synthesis. Although the model of Huang et al. (7) (Fig. 1A) depicts important interactions between the fingers subdomain of HIV-1 RT and the single-stranded template overhang, their significance during dNTP catalysis is unclear. Our nucleoside analog strategy sheds light on the importance of interactions between Lys-154, Arg-78, and Trp-24 with phosphates n/H11002, n/H11001, and n/H110012/H110013, respectively, of the template backbone as well as between Leu-74 with nucleobase n/H110011 and Phe-61 with nucleobase n/H110012 and ribose n/H110011.

Incorporation opposite an abasic lesion was significantly reduced by replacing amino acids proposed to interact with the template overhang (F61A and F61L RT) or eliminating adjacent 5/2 template nucleobases (wild type and F61T RT, Figs. 4A and 5A). Because THF lesions preserve the sugar-phosphate backbone, the inability of F61A and F61L RT to incorporate opposite the abasic site supports an important role for Phe-61 (together with Gly-152 and Leu-74) in contacting template nucleobase n/H110011 (7) but provides no information on the involvement of the adjacent 5/2 nucleobase. Data in Figs. 4A and 5A suggest that, provided the appropriate contacts are made with nucleobase +2 (clearly requiring residues with an aromatic side chain at position 61), this can compensate for eliminating nucleobase n + 1, allowing correct positioning and incorporation of the incoming dNTP. The same model predicts...
that simultaneously removing template nucleobases $n + 1$ and $n + 2$ disrupts critical contacts with Phe-61, which would affect incorporation opposite the initial lesion. Experimentally this phenotype was observed with wild type RT and p66 mutant F61T.

The response of wild type RT and in particular mutants F61A and F61L to LNA analogs under running start conditions (Fig. 3B) illustrates the importance of template geometry nt ahead of the catalytic center. NMR studies with single-stranded LNA-containing oligonucleotides indicate the conformationally restricted sugar increases backbone organization (38). In the HIV-1 RT structure of Huang et al. (7), the template overhang bends away from the duplex, with Phe-61 contacting nucleobase $n + 2$ and Trp-24 contacting the $n + 2/n + 3$ phosphate. Thus, LNA-induced alterations in backbone organization might alter contacts such that the template overhang no longer follows its natural trajectory, halting DNA synthesis. Alternatively, because LNA analogs are known to affect neighboring residues (38), we must also consider the possibility that an $n + 2$ substitution could affect how nucleobase $n + 1$ is wedged beneath the side chain of Leu-74 and the backbone of Gly-152 (7). As the number of LNA substitutions 5' to the initial analog increased, their additive effect on template flexibility results in increased pausing by F61A and F61L RT after dNTP incorporation opposite template nucleotide $n + 6$. The insensitivity of F61T RT to multiple template LNA insertions (Figs. 3B and 5B) was surprising. Previous studies indicated unaltered processivity for this RT mutant (35). Thus, the phenotype of Figs. 3B and 5B cannot be explained simply in terms of enhanced processivity. Conceptually, enhanced stacking interactions of the indole ring of Trp may permit this mutant to more rapidly shuttle between the priming and nucleotide binding sites described by Boyer et al. (56), effectively enhancing translocation across the lesion. Stalling of wild type RT after incorporation opposite a nucleobase 5' to a methylphosphonate linkage during both running start and standing start conditions (Figs. 2C and 4C, respectively) supports an important role for Lys-154 in contacting the template $n - 1/n$ phosphate (Fig. 1A). Enhanced stalling at the same site when the template $n - 1/n$ and $n/n + 1$ phosphates were simultaneously neutralized implicate Arg-78 in contacting the $n/n + 1$ phosphate, which is also consistent with structural data (7). Surprisingly, three consecutive methylphosphonate linkages further enhanced stalling at the same position. Such a result implicates contact between residues of the p66 fingers subdomain and the template $n + 1/n + 2$ phosphate, for which no contact is evident from crystallography.

Finally, primer extension beyond the position of analog insertion indirectly probes how a lesion-containing duplex traverses the nucleic acid binding cleft. For example, data of Figs. 2C and 4C indicate wild type HIV-1 RT pauses after dNTP incorporation opposite a nucleobase 5' to a single template methylphosphonate substitution. However, although DNA synthesis thereafter is reduced, additional sites where pausing was selectively enhanced were absent despite the same linkage occupying between positions $n - 1$ and $n - 11$ of duplex DNA in the polymerization complex. Continued synthesis beyond the lesion predicts that contact between the template backbone and Glu-89 ($n - 1/n - 2$ phosphate), Lys-374 ($n - 4/n - 5$ phosphate), Lys-353 ($n - 5/n - 6$ phosphate), and Arg-285 ($n - 6/n - 7$ and $n - 7/n - 8$ phosphates) would be “probed” by the same lesion. Our data suggest that critical template backbone contacts are restricted exclusively to those at or immediately ahead of the catalytic center. LNA-containing templates provide a second example where duplex DNA with an altered geometry over as much as three base pairs traverses uninterrupted between positions –1 and –11 of the polymerization complex (Figs. 2B and 4B). The length of the templates used here did not allow us to study how the same lesions affected DNA synthesis as they traversed the C-terminal ribonuclease H domain but illustrate the importance of nucleoside analogs in fine structure analysis of protein-nucleic acid contacts.

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doi: 10.1074/jbc.M603970200 originally published online July 25, 2006

Access the most updated version of this article at doi: 10.1074/jbc.M603970200

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